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(54) Title: METHOD FOR PREPARING A (R)- OR (S)- FORM OF N-(2,6-DIMETHYL PHENYL) ALANINE AND A COUNTER ENANTIOMERIC FORM OF N-(2,6-DIMETHYL PHENYL) ALANINE ESTER THERETO USING ENZYME

(57) Abstract: The present invention provides methods for stereospecifically preparing (R)- or (S)-N-(2,6-dimethyl phenyl) alanine and counter ester compounds thereto using enzyme, which comprises reacting an enzyme having hydrolytic activity specific to one enantiomer of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine esters ((R)-enantiomer and (S)-enantiomer) with the racemic mixture above to obtain (R)- or (S)-N-(2,6-dimethyl phenyl) alanine by optical resolution, or extracting (S)- or (R)-N-(2,6-dimethyl phenyl) alanine ester as an unreacted compound from a reaction mixture using solvent, or esterifying a certain alanine enantiomer separated by the optical resolution with alcohol to synthesize (S)- or (R)-N-(2,6-dimethyl phenyl) alanine ester.

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METHOD FOR PREPARING A (R)- OR (S)- FORM OF N-(2,6-DIMETHYL PHENYL) ALANINE AND A COUNTER ENANTIOMERIC FORM OF N-(2,6-DIMETHYL PHENYL) ALANINE ESTER THERETO USING ENZYME

FIELD OF THE INVENTION

The present invention relates to methods for stereospecifically preparing (R)- or (S)-N-(2,6-dimethyl phenyl) alanine and counter ester compounds thereto using enzyme, more specifically, methods of comprising reacting an enzyme having hydrolytic activity specific to one kind of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine esters ((R)-enantiomer and (S)-enantiomer) with the racemic mixture above to obtain (R)- or (S)-N-(2,6-dimethyl phenyl) alanine by optical resolution, or extracting (S)- or (R)-N-(2,6-dimethyl phenyl) alanine ester as an unreacted compound from a reaction mixture using solvent, or esterifying a certain alanine enantiomer separated by the optical resolution with alcohol to synthesize (S)- or (R)-N-(2,6-dimethyl phenyl) alanine ester.

BACKGROUND OF THE INVENTION

Racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine and their ester compounds are useful as precursors for the synthesis of Metalaxyl, Benalaxyl, Furalaxyl, etc. having antifungal activity. The antifungal activity of these compounds is generally resides with the (R)-enantiomer.

Synthesis of Metalaxyl-M which is (R)-enantiomer of Metalaxyl has been reported to be achieved by hydrogenation of enamide in the presence of a chiral metallic catalyst (*Pesticide Science*, Vol. 54, 1998, pp 302-304). This reaction provides Metalaxyl-M in 95.6% ee (% enantiomeric excess) but requires an expensive metallic catalyst and a releasing process such as

treatment under high temperature and high pressure.

As one approach for preparation of an optically active compound, (R)- or (S)-N-(2,6-dimethyl phenyl) alanine, an optical resolution has been known employing optically pure amine as resolving agent, for example, (R)- or (S)-phenylethylamine to separate the racemic mixture (U.S. Patent No. 4,919,709). However, this method has some demerits that the above resolving agent is expensive and the process therefor is complicated.

As one approach for synthesis of (R)-N-(2,6-dimethyl phenyl) alanine methyl ester, it has been reported that a sulfonate derivative is made starting from methyl (S)-2-(hydroxy)-propanoate and then reacted with 2,6-dimethyl aniline in the presence of base (WO 00/76960). However, according to this method, it is difficult to obtain (R)-N-(2,6-dimethyl phenyl) alanine methyl ester with enantiomeric excess greater than 95% ee, and high temperature is required for performance of the process. And also (R)-N-(2,6-dimethyl phenyl) alanine methyl ester can also be applied to the chiral form of Benalaxyl, Furalaxyl, etc.; however, the required optical purity for Metalaxyl-M is more than 95% ee.

Meanwhile, for optical resolution of racemic mixtures, the use of enzymes such as esterases, lipases and proteases has been known which selectively hydrolyze enantiomers. For example, hydrolyzing racemic methyl-2-chloropropionates with lipase isolated from *Candida rugosa* has been reported (Biotechnology & Bioengineering 30, 1987, pp 995-999). Moreover, synthesizing (R)-2-(4-hydroxyphenoxy) propionic acid with purified *Candida rugosa* lipase has been reported (WO 90/15146).

While the use of enzymes for the optical resolution of racemic mixtures is very effective, it is very difficult to determine which racemic mixtures can be applied to enzymatic resolution and which enzymes can be employed for the optical resolution of a specific racemic

mixture. For example, inventors of U.S. Patent No. 5,928,933 performed experiments for optical resolution of racemic 4-oxo-1,2-pyrrolidine dicarboxylic acid dialkyl esters using about forty-four different enzymes chosen from proteases, lipases and esterases, and found only one enzyme yielding an optical purity of 95%. Like this case, the optimal choice of enzyme and substrate is not easily predicted, but requires a careful screening of a variety of enzymes while varying the chemical structure of potential substrates.

Meanwhile, with respect to racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine of the present invention, no attempts using any enzymes for the optical resolution thereof have been made. The existing optical resolutions using enzymes have been limited to the synthesis of aryloxypropionic acid as a precursor for crop-based herbicide and the synthesis of arylpropionic acid as a precursor for profen-based antiinflamatory agent. In other words, there have not yet been any reports of using enzymes for the optical resolution of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine ester.

SUMMARY OF THE INVENTION

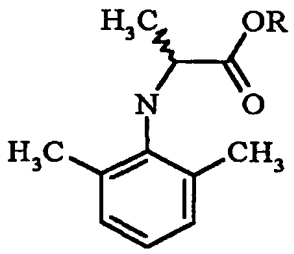
The inventors of the present invention have carried out extensive investigations and thereby determined that when the racemic mixture of (R), (S)-N-(2,6-dimethyl phenyl) alanine ester is reacted under room temperature and atmospheric pressure with an enzyme hydrolysizing only one of both enantiomers in the racemic mixture, (R)- or (S)-N-(2,6-dimethyl phenyl) alanine and its counter ester can be prepared at high optical purity (> 96-99% ee). The present invention is achieved based upon this finding.

Accordingly, the object of the present invention is to provide a method of economically preparing (R)- or (S)-N-(2,6-dimethyl phenyl) alanine and (R)- or (S)-N-(2,6-dimethyl phenyl)

alanine ester at high optical purity by optical resolution using enzyme.

According to an aspect of the present invention, the method of preparing (R)- or (S)-N-(2,6-dimethyl phenyl) alanine comprises steps of,

(A) reacting racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine ester as represented in Formula 1 below,



(1)

where

R is selected from the group consisting of unsubstituted or substituted and linear or branched C_1-C_{18} alkyl or alkenyl, unsubstituted or substituted C_3-C_6 cycloalkyl, unsubstituted or substituted aryl alkyl, and unsubstituted or substituted heteroaryl alkyl,

with an effective amount of an enzyme which enantioselectively hydrolyzes one enantiomeric form thereof to produce an (R)-form or (S)-form of N-(2,6-dimethyl phenyl) alanine ("(R)-form alanine" or "(S)-form alanine") and a counter enantiomeric form of N-(2,6-dimethyl phenyl) alanine ester ("counter (S)-form ester" or "counter (R)-form ester"); and

(B) isolating the (R)-form or (S)-form alanine from a reaction mixture to obtain an optically pure N-(2,6-dimethyl phenyl) alanine.

R in Formula 1 includes, but is not limited to, for example, methyl, ethyl, n-propyl,

isopropyl, n-butyl, 2-butyl, 2-pentyl, 3-methyl-1-butyl, 2-ethyl-1-hexyl, 2-chloroethyl, 2-bromoethyl, 3-chloropropyl, 3-bromopropyl, 2,2-dichloroethyl, 1-chloro-2-propyl, oleyl, cyclohexyl, 1-cyclopropylmethyl, allyl, phenyl, benzyl, propargyl, 2-phenoxy-1-ethyl, 2,4-dichlorobenzyl, methoxyethyl, ethoxyethyl, 1-thioethoxyethyl, and the like.

According to another aspect of the present invention, a method of preparing (R)- or (S)-N-(2,6-dimethyl phenyl) alanine ester comprises the steps of,

(A') reacting racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine ester as represented in Formula 1 above with an effective amount of an enzyme which enantioselectively hydrolyzes one enantiomeric form thereof to produce an (R)-form or (S)-form of N-(2,6-dimethyl phenyl) alanine ("(R)-form alanine" or "(S)-form alanine") and a counter enantiomeric form of N-(2,6-dimethyl phenyl) alanine ester ("counter (S)-form ester" or "counter (R)-form ester"); and

(B') isolating the unhydrolyzed counter (S)-form or (R)-form ester from a reaction mixture to obtain an optically pure N-(2,6-dimethyl phenyl) alanine ester.

In an embodiment, the step (B') may be replaced with the following step (B"):

(B") isolating the created (R)-form or (S)-form alanine from a reaction mixture and then esterifying the (R)-form or (S)-form alanine with an alcohol, R-OH, wherein R is the same as in Formula 1, to produce an optically N-(2,6-dimethyl phenyl) alanine ester.

DETAILED DESCRIPTION OF THE INVENTION

Some terms and abbreviations used in this disclosure are defined as the below.

The term "racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine ester" is used to express the racemic mixture of (R)-N-(2,6-dimethyl phenyl) alanine ester and (S)-N-(2,6-dimethyl

phenyl) alanine ester.

The term "enantiomer" is used to express one of a pair of molecular entities which are mirror images of each other and non-superimposable. The term "enantiomer" is employed to sometimes express an enantiomeric alanine and sometimes express an enantiomeric alanine ester, depending upon contexts in this disclosure.

The term "counter" is used to express the corresponding ester of a complementary enantiomeric alanine to a certain enantiomeric alanine; i.e., the counter ester to (R)-N-(2,6-dimethyl phenyl) alanine means (S)-N-(2,6-dimethyl phenyl) alanine ester, and the counter ester to (S)-N-(2,6-dimethyl phenyl) alanine means (R)-N-(2,6-dimethyl phenyl) alanine ester.

For convenience of illustration, the term "(R)-N-(2,6-dimethyl phenyl) alanine" is sometimes abbreviated as "(R)-form alanine" and the term "(S)-N-(2,6-dimethyl phenyl) alanine" is sometimes abbreviated as "(S)-form alanine." Likewise, the term "(R)-N-(2,6-dimethyl phenyl) alanine ester" is sometimes abbreviated as "(R)-form ester" and the term "(S)-N-(2,6-dimethyl phenyl) alanine ester" is sometimes abbreviated as "(S)-form ester." In some cases, the term "(R)-form enantiomer" or "(S)-form enantiomer" is used and its meaning, i.e., whether it is used to express (R)-form alanine or (R)-form alanine ester, can be easily understood in the related contexts.

Methods for preparing racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine ester, to which the optical resolution is carried out according to the present invention, are well known in the art to which the present invention pertains. As one example, the racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine ester can be readily synthesized by reaction of 2-bromopropionic acid ester and 2,6-dimethylaniline. However, it is very difficult to isolate only (R)-form enantiomer or (S)-form enantiomer from the racemic mixture of (R), (S)-N-(2,6-dimethyl phenyl) alanine ester

because both enantiomers have very similar physical and chemical properties.

Therefore, relative to previously known methods for optical resolution by chemical synthesis or separation, the present invention uses an enzyme which enantioselectively hydrolyzes only one of (R)-form enantiomer [(R)-N-(2,6-dimethyl phenyl) alanine ester] and (S)-form enantiomer [(S)-N-(2,6-dimethyl phenyl) alanine ester] (hereinafter, sometimes referred to as "specific hydrolysis enzyme"), thereby allowing a particular enantiomer to be readily synthesized by a simple and inexpensive process.

In the step (A) of the method for preparation of (R)- or (S)-N-(2,6-dimethyl phenyl) alanine, the racemic ester mixture is dissolved in an aqueous solution or a mixed solution consisting of water and a small amount of organic solvent and then reacted with a specific hydrolysis enzyme at a uniform temperature and pH.

Where a specific hydrolysis enzyme enantioselectively hydrolyzes (R)-form enantiomer, only (R)-form ester is hydrolyzed in the reaction of the step (A) such that (R)-N-(2,6-dimethyl phenyl) alanine is created and (S)-form ester, i.e., (S)-N-(2,6-dimethyl phenyl) alanine ester remains unreacted. Conversely, where a specific hydrolysis enzyme enantioselectively hydrolyzes (S)-form enantiomer, only (S)-form ester is hydrolyzed in the reaction of the step (A) such that (S)-N-(2,6-dimethyl phenyl) alanine is created and (R)-form ester, i.e., (R)-N-(2,6-dimethyl phenyl) alanine ester remains unreacted.

Isolation of the hydrolyzed enantiomer from the reaction mixture in the step (B) can be achieved in different ways, depending upon a reaction system. Where the reaction system consists of only aqueous solution, an unhydrolyzed ester compound ((R)- or (S)-form ester) can be extracted using organic solvents. Where the reaction system consists of a mixed solution of organic solvent and aqueous solution, an organic layer containing an unhydrolyzed ester

compound can be readily partitioned to obtain an aqueous layer containing newly synthesized alanine because the unhydrolyzed ester compound tends to be soluble in organic solvents.

(R)- or (S)-form alanine which is contained in the aqueous solution or in the aqueous layer is acidified and then extracted using an organic solvent, whereafter the organic solvent is removed to obtain the optically pure (R)- or (S)-N-(2,6-dimethyl phenyl) alanine. From various experiments, (R)- or (S)-N-(2,6-dimethyl phenyl) alanine obtained by the methods according to the present invention was ascertained to have a very high optical purity (> 96 - 99% ee).

Likewise, (R)- or (S)-N-(2,6-dimethyl phenyl) alanine ester was also ascertained to have a very high optical purity (> 96 – 99% ee), which was separated in the unreacted form from the reaction mixture in the step (B'), or synthesized by esterification of the synthesized (R)- or (S)-N-(2,6-dimethyl phenyl) alanine with the alcohol in the step (B").

R in Formula 1 is desirably selected from the group consisting of allyl, 2-chloroethyl, methoxyethyl and ethoxyethyl because these groups can allow a short reaction time and increased optical purity.

The kinds of specific hydrolysis enzymes are not particularly limited so long as they can enantioselectively hydrolyze only one of (R)-form ester and (S)-form ester, but are preferably selected from lipases, proteases and esterases derived from microorganisms, animals or plants.

Among them, lipases are preferable, providing a high enantioselectivity and good optical purity. More preferably, the specific hydrolysis enzyme is one or more selected from the group consisting of Lipase AK from *Pseudomonas*, Toyobo Immobilized lipase, Lipoprotein lipase, Lipase PS and AH from *Burkhoderia*, Lipase QLM from *Alcaligenes*, and Lipase OF

from *Candida*, which are particularly good in view of their enantioselectivity and reaction rate.

Selectivity to (R)-form and (S)-form enantiomers is determined by the kind of enzymes used in the hydrolysis reaction. Enzyme may be applied to the reaction system in the form of powder or aqueous solution. In some embodiments, a support-immobilized enzyme can be employed to simplify catalyst recovery for reuse. Such immobilization methods, e.g., attaching enzymes onto polymer support or inorganic support such as celite, are well known to those skilled in the art, and the detailed description thereof is therefore omitted.

Since the effective amount of enzyme depends upon the reaction parameters, for example, the reaction temperature, pH, concentration, reaction time, etc., its addition amount may be variably determined. In some embodiments, the effective amount of enzyme is preferably in the range of 0.1 – 100% by weight based upon the weight of substrate (racemic ester mixture). If the amount of enzyme used in the reaction is below the minimum effective amount, the reaction time may be undesirably extended, and if the amount of enzyme is above a certain maximum effective value, the cost of the process is undesirably increased by the use of excessive enzyme and more extensive separation processes.

The reaction conditions are not particularly limited but, for optimization of the enzymatic reaction, are preferably in the range of pH 3 – 12 and temperature 0 – 60°C, more preferably 30 – 50°C. As mentioned above, the hydrolysis reaction of step (A) may be carried out in an aqueous solution, or a mixed solution containing a small amount of organic solvent so as to enhance the solubility of substrate and reduce the inhibition of enzyme activity by reaction products.

The concentration of substrate used in the enzymatic reaction may also be varied depending upon various reaction parameters. Preferably, it is in the range of 500 mM – 1M for

optimization of the enzymatic reaction but may be beyond 1 M in some cases. In the hydrolysis of step (A) and (A'), ester compounds of the racemic mixture may not be well dissolved in an aqueous solution described in the initial procedure, but some ester compounds dissolved in the aqueous solution contact and react with the enzyme. In order to increase the reaction rate and maintain the activity of enzyme, a reaction system consisting of a small amount of water and a large amount of organic solvent may be used.

Examples of the organic solvent used in the enzymatic reaction include, but are not limited to, hydrophilic solvents such as acetone, acetonitrile, alcohol, etc. and hydrophobic solvents such as isopropyl ether, tert-butyl methyl ether, chloroform, dichloromethane, carbon tetrachloride, hexane, toluene, etc. In some embodiments, a two-phase solvent consisting of hydrophilic and hydrophobic solvents can also be employed.

Examples of the organic solvent used in the extraction of step (B) or (B') include, but are not limited to, ethyl acetate, isopropyl ether, tert-butyl methyl ether, chloroform, dichloromethane, carbon tetrachloride, hexane, toluene, etc. and the mixture of two or more thereof.

The esterification reaction in the step (B") is not particularly limited so long as it can be accomplished by reaction between the carboxyl group of (R)- or (S)-N-(2,6-dimethyl phenyl) alanine and alcohol to give the desired (R)- or (S)-N-(2,6-dimethyl phenyl) alanine ester, which can be easily achieved by those skilled in the art, thus the description thereof is omitted in this disclosure.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Hereinafter, the present invention will be described in more detail by EXAMPLES, but

the scope of the present invention is not limited thereto.

EXAMPLE 1: Enzyme screens for optical resolution of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine methyl ester

A substrate solution containing 50 μl of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine methyl ester in 500 μl of acetonitrile was added to 450 μl of potassium phosphate buffer and 25 mg of enzyme powder (in the case of enzyme solution, corresponding to 20 μl of enzyme solution) was then added thereto. Reactions were run at 30°C for 1 and 2 days. The conversion rate of reaction was analyzed by HPLC on a C18 reverse phase column with a mixture of acetonitrile and water (acetonitrile : water = 70 : 30, 0.1% trifluoroacetic acid) as the eluting solvent while detecting at 254 nm wavelength. The kinds of enzymes used and the conversion rates of the racemic mixture are listed in TABLE 1 below.

TABLE 1

enzyme	source	conv.(%)	
		1 day	2 day
Optimase	*Bacillus licheniformis*	61	83
Alcalase	*Bacillus licheniformis*	60	83
Lipase PS	*Burkholderia cepacia*	50	56
Novozym 435	*Candida antarctica*	100	-
Lipase OF	*Candida rugosa*	47	57
Lipase PL	*Alcaligenes* sp.	32	49
Lipase PS-D	*Burkholderia cepacia*	53	61
Lipase PS-C	*Burkholderia cepacia*	56	60
Lipase AL	*Achrobacter* sp.	19	23
Lipase QLM	*Alcaligenes* sp.	60	76
Proleather FG-F	*Bacillus subtilis*	56	79
Acylase Amano	*Aspergillus melleus*	51	65
Protease PS	*Bacillus sp.*	100	-
Immobililized lipase	*Pseudomonas* sp.	45	50
Lipoprotein lipase	*Pseudomonas* sp.	61	69
Lipase AH	*Burkholderia cepacia*	56	62
PLE-AL amano	Porcine liver	100	-
Alcalase 0.6L	*Bacillus licheniformis*	35	48

Alcalase 2.5L	*Bacillus licheniformis*	65	84
Novozym 525L	*Candida antarctica*	100	-
ChiroCLEC-CAB	*Candida antarctica*	100	-

In TABLE 1 above, enzymes showing a conversion rate of around 50% after the lapse of a certain reaction time are potential candidates as highly enantioselective enzymes specific to (R)-form enantiomer or (S)-form enantiomer. In this view, a variety of lipases can be considered as such candidates having a high enantioselectivity.

EXAMPLE 2: Enzyme screens for optical resolution of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine methyl ester

A racemic mixture of enriched (chemical synthesized) (R)-N-(2,6-dimethyl phenyl) alanine methyl ester (R : S = 90 : 10) and a racemic mixture of enriched (chemical synthesized) (S)-N-(2,6-dimethyl phenyl) alanine methyl ester (R : S = 4 : 96) were prepared respectively, and the conversions rates were measured in the same manner as in EXAMPLE 1. The kinds of enzymes used in experiment and the conversion rates of the racemic mixtures are listed in TABLE 2 below.

TABLE 2

enzyme	Source	conv.(%)	
		(R)-ester	(S)-ester
Lipase AK	*Pseudomonas fluorescens*	79	17
Lipase AH	*Burkholderia cepacia*	47	12
Lipase PS	*Burkholderia cepacia*	86	17
Lipase QLM	*Alcaligenes sp.*	96	19

From the results in TABLE 2 above, it can be seen that Lipase AK, PS and QLM have high enantioselectivity specific to (R)-form enantiomer.

EXAMPLE 3: Enzyme screens for optical resolution of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine allyl ester and racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine 2-chloroethyl

ester

2 mg of enzyme powder was suspended in 1 ml of 0.1 M potassium phosphate buffer (pH 7.0) and 20 mg of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine allyl ester was then added thereto, followed by shaking of the resulting mixture at 200 rpm and 35°C. After 3 and 24 hours, respectively, 20 μ of sample was dissolved in a mixed solution of hexane and isopropyl alcohol (hexane : isopropyl alcohol = 95 : 5) and adjusted to pH 10 using 5% Na_2CO_3. The unreacted ester compound was extracted to the organic layer (organic solvent). The aqueous layer was adjusted to pH 3 – 4 using 1 N HCl, then was extracted with ethyl acetate. After partitioning the layers, organic solvents were removed by vacuum distillation and the residue was again dissolved in a mixed solution of hexane and isopropyl alcohol (hexane : isopropyl alcohol = 95 : 5) to analyze the enantioselectivity.

The above procedure was repeated in the same manner except for using (R), (S)-N-(2,6-dimethyl phenyl) alanine 2-chloroethyl ester instead of the above racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine allyl ester.

The analysis conditions are the following:

Chiracel OD column, 30°C, 0.8 ml, 254 nm

Acid analysis – hexane : isopropyl alcohol : trifluoroacetic acid = 95 : 5 : 0.1

Ester analysis – hexane : isopropyl alcohol = 100 : 1

The analysis results are listed in TABLE 3 below.

TABLE 3

Enzyme	allyl ester				2-chloroethyl ester			
	conv.(%)		% ee_p(R)		conv.(%)		% ee_p(R)	
	3 hr	24 hr	3 hr	24 hr	3 hr	24 hr	3 hr	24 hr
Lipase AK	33.5	50.3	99.4	98.8	49.2	-	97.8	-
Lipase GF	-	23.1	-	100	-	14.0	-	98.2

Lipase AG	-	16.7	-	50	-	-	not selective	
Lipase AH	20.6	50.2	100	99.2	49.8	-	98.6	-
Novozym 435	not selective							
Esterase CCE	-	66.7	-	4	not selective			
Esterase PPE	-	2.3	-	85.4	-	4.1	-	91.8
Lipase TL	14.4	53.2	94.8	80.8	53.5	-	86.3	-
Lipase UL	-	2.0	-	100	4.1	6.2	93	22
Lipase AL	-	3.8	-	100	6.2	25.1	91.4	95.6
Lipase QLM	41.4	49.7	99.0	97.0	-	60.8	-	62
Immobilized lipase (Toyobo)	7.7	47.7	95.8	96.6	22.3	52.7	90.4	70
Lipase BG	-	-	-	-	-	46.1 (17 hr)	-	91.5 (17 hr)

conv. = $ee_s / (ee_s + ee_p)$

wherein conv. means conversion, and ee_p and ee_s mean the optical purity of product and the optical purity of reactant, respectively, as represented by the below formulas.

ee_s = [(R)-N-(2,6-dimethyl phenyl) alanine ester – (S)-N-(2,6-dimethyl phenyl) alanine ester] / [(R)-N-(2,6-dimethyl phenyl) alanine ester + (S)-N-(2,6-dimethyl phenyl) alanine ester]

ee_p = [(R)-N-(2,6-dimethyl phenyl) alanine – (S)-N-(2,6-dimethyl phenyl) alanine] / [(R)-N-(2,6-dimethyl phenyl) alanine + (S)-N-(2,6-dimethyl phenyl) alanine]

As can be seen in TABLE 3, some lipases have a high conversion rate of around 50% and good enantioselectivity in both the racemic mixture of allyl ester and the racemic mixture of 2-chloroethyl ester, and the optimal reaction times vary depending upon the kind of esters.

EXAMPLE 4: Optical resolution of (R), (S)-N-(2,6-dimethyl phenyl) alanine 2-chloroethyl ester by Lipase OF

20 mg of a racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine 2-chloroethyl ester was added to 1 ml of 1 M potassium phosphate buffer (pH 7.0) and 2 mg of Lipase OF was then added thereto, followed by shaking of the resulting mixture at 200 rpm and 35°C. The analysis of reaction was carried out in the same manner as in EXAMPLE 3. After 17 hrs, (S)-N-(2,6-dimethyl phenyl) alanine of 70.8% ee was obtained at 41.4% conversion. Since *Candida rugosa* Lipase OF comprises mixed forms of a variety of isozymes, use of a specific isozyme among them is anticipated to be able to increase the optical purity of product.

EXAMPLE 5: Reactivity of enzyme depending upon the kind of esters

20 mg of a variety of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine esters were dissolved in 1 ml of 0.1 M potassium phosphate buffer (pH 7.0), respectively, and 2 mg of Lipase PS was added thereto, then the analysis was performed in the same manner as in EXAMPLE 3. The analysis results are listed in TABLE 4 below.

TABLE 4

Ester	conv.(%)		% ee_p(R)	
	3 hr	24 hr	3 hr	24 hr
2-chloroethyl	48.3	52.6	97.0	90.2
2-bromoethyl	36.5	62.1	84.4	21.6
3-chloropropyl	12.2	48.0	99.2	99.4
3-bromopropyl	6.2	38.8	100	100
2,2-dichloroethyl	35.5	51.8	98.6	93.2
1-chloro-2-propyl	-	10.7	-	100
methoxyethyl	39.6	52.1	99.0	92.0
ethoxyethyl	48.1	51.2	98.8	95.4
1-thioethoxyethyl	10.0	34.4	88.0	96.8
isopropyl	-	11.2	-	86.8
2-butyl	-	12.7	-	100
2-pentyl	-	9.2	-	61.4
3-methyl-1-butyl	-	25.3	-	100
2-ethyl-1-hexyl	-	28.4	-	96.8
Benzyl	-	<10	-	96.6
2-phenoxy-1-ethyl	14.7	37.9	97.6	95.8

2,4-dichlorobenzyl	-	4.1	-	65
oleyl	-	12.0	-	98.4
allyl	32.5	50.5	99.6	98.0
propargyl	42.8	53.5	96.6	87.0
methyl	17.1	41.5	96.6	97.2
ethyl	37		98.6	-
n-propyl	37		97.6	-
n-butyl	33.9		98.4	-
cyclohexyl	0.26		23.2	-
1-cyclopropylmethyl	-	8.8	-	99.0
Phenyl	-	-	23.2	-
trifluoroethyl	-	-	-	not selective
n-butanethioester	-	-	45.0	-

Conv. = $ee_s/(ee_s + ee_p)$

As can be seen in TABLE 4 above, 2-chloroethyl, 2-bromoethyl, allyl, methoxyethyl, ethoxyethyl ester and the like are particularly preferable as substrates for Lipase PS.

EXAMPLE 6: Optical resolution of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine 2-chloroethyl ester

500 mg of a racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine 2-chloroethyl ester was added to 2.5 ml of potassium phosphate buffer (pH 7.0) (concentration: 200 g/l) and 10 mg of enzyme was then added thereto (ester : enzyme = 50 : 1), followed by shaking the resulting mixture at 200 rpm and 35°C. The analysis of reaction was performed in the same manner as in EXAMPLE 3. The analysis results are listed in TABLE 5 below.

TABLE 5

Enzyme	Source	conv.(%)			% ee_p(R)		
		6hr	24hr	36hr	6hr	24hr	36hr
Lipaes AH	*Burkholderia cepacia*	6.2	21.1	31.5	96.0	98.8	98.2
Lipase AK	*Pseudomonas fluorescens*	16.8	36.8	45.4	96.8	98.8	97.2
Lipase PS	*Burkholderia cepacia*	21.2	40.7	45.3	98.6	98.6	95.2

Lipase QLM	*Alcaligenes sp.*	25.0	44.0	47.1	98.4	97.0	94.8
Lipase TL	*Pseudomonas stutzeri*	25.9	41.6	44.8	94.4	90.4	88.4

Conv. = $ee_s/(ee_s + ee_p)$

The results of TABLE 5 above show that the above-referenced racemate can be enantioselectively separated by the enzymes listed therein at an excellent conversion rate and optical purity. Furthermore, Lipase AK, PS and QLM provide a very high conversion rate approaching 50%.

EXAMPLE 7: Optical resolution of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine ester varies with ratio of substrate and enzyme

1 g of several racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine esters was added to 2.5 ml of 0.5 M potassium phosphate buffer (pH 7) (concentration: 400 g/L) and varying amounts of enzyme were then added thereto, respectively, followed by shaking the resulting mixture at 200 rpm and 35°C. The analysis of the reaction was carried out in the same manner as in EXAMPLE 3. The analysis results are listed in TABLE 6 below.

TABLE 6

Lipase PS	2-chloroethyl ester				methoxyethyl ester				ethoxyethyl ester			
amount	conv.(%)		% ee_p(R)		conv.(%)		% ee_p(R)		conv.(%)		% ee_p(R)	
(ester:enzyme)	1d.	2d.	1d.	2d.	1d.	2d.	1d.	2d.	1d.	2d.	1d.	2d.
10mg (100:1)	18.2	31.2	98.6	98.0	15.7	30.3	98.4	96.8	15.5	26.2	99.2	99.0
20mg (50:1)	29.4	39.3	98.4	97.6	28.7	38.1	98.2	97.6	22.5	31.1	99.0	98.4
50mg (25:1)	35.5	41.9	98.2	96.6	40.6	45.0	97.4	98.2	28.0	39.4	98.6	97.8

Conv. = $ee_s/(ee_s + ee_p)$

As seen in TABLE 6 above, it can be seen that the conversion rate increases as the amount of enzyme used increases. It can also be seen that the optimal amount of enzyme should

be determined considering various parameters such as the kinds of enzyme and ester, conversion rate, optical purity and the like.

EXAMPLE 8: Optical resolution of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine methyl ester by immobilized Lipase PS

0.1 g of Lipase PS was dissolved in 10 ml of 0.1 M calcium phosphate buffer (pH 7.0) and the resulting mixture was maintained at room temperature with being stirred, followed by filtering to remove insoluble materials. 4.5 ml of the filtrate solution was mixed with 1 M potassium phosphate (pH 7.0) and 30 mg of Sepabeads FP-EP16® (Resindion) was then added thereto, followed by stirring at room temperature overnight and subsequent filtering. The bead-immobilized enzyme obtained thus was added to 1 ml of 0.1 M Tris buffer (pH 7), 10 mg of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine methyl ester was added thereto, then the reaction was run at 30°C for 17 hours while being shaken at 200 rpm. The analysis of reaction was performed at the same manner as in EXAMPLE 3.

The above procedure was repeated except for using Eupergit C® (Rohm & Haas) as a support for enzyme immobilization.

In the case of Sepabeads FP-EP16®, (R)-N-(2,6-dimethyl phenyl) alanine of 98.5% ee was obtained at 25% conversion. In the case of Eupergit C®, (R)-N-(2,6-dimethyl phenyl) alanine of 98.4% ee was obtained at 46% conversion.

EXAMPLE 9: Optical resolution of (R), (S)-N-(2,6-dimethyl phenyl) alanine 2-chloroethyl ester by immobilized Lipase PS

0.1 g of Lipase PS was dissolved in 5.0 ml of 0.1 M sodium phosphate buffer (pH 6.0)

and, while being stirred, XAD®-7HP (Rohm & Haas) was added thereto at varying amounts of 100 mg (resin : enzyme = 1 : 1), 250 mg, 500 mg and 1.0 g. The resulting mixture was stirred at 30°C for 24 hours and then filtered. The resin and insoluble materials were added to 0.1 M sodium phosphate buffer (pH 6.0) and 5 μl of glutaraldehyde was also added thereto, followed by stirring at room temperature for 3 hrs. The immobilized enzyme (corresponding to 10 mg of Lipase PS) obtained thus was added to 1 ml of 0.5 M potassium phosphate buffer (pH 8) and 100 mg of (R), (S)-N-(2,6-dimethyl phenyl) alanine 2-chloroethyl ester was then added thereto, followed by shaking at 200 rpm, 35°C for 16 hrs. to allow the reaction to proceed. The analysis of the reaction was performed in the same manner as in EXAMPLE 3. The analysis results are listed in TABLE 7 below.

TABLE 7

Lipase PS	XAD-7HP amount	conv.(%)	% ee_p(R)
100 mg	100 mg	16	87.0
100 mg	250 mg	23	93.0
100 mg	500 mg	40	95.6
100 mg	1000 mg	49	96.3

Conv. = $ee_s/(ee_s + ee_p)$

EXAMPLE 10: Optical resolution of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine methyl ester in mass production

20 ml of (R), (S)-N-(2,6-dimethyl phenyl) alanine methyl ester (concentration: 400 g/L) was added to 30 ml of distilled water and 500 mg of Lipase PS was then added thereto, followed by stirring (30%, Mettler DL70 titrator) at 40°C to run a reaction. In order to maintain uniform pH of the reaction mixture, 1 N NaOH was automatically added thereto (Mettler DL70 titrator). After 24 hrs, 250 mg of Lipase PS was additionally added thereto. The analysis of the reaction was carried out in the same manner as in EXAMPLE 3. (R)-N-(2,6-dimethyl phenyl)

alanine of 96.9% ee was obtained at 40.2% conversion (after 6 days).

EXAMPLE 11: Optical resolution of (R), (S)-N-(2,6-dimethyl phenyl) alanine methyl ester by enzyme and isolation of (R), (S)-N-(2,6-dimethyl phenyl) alanine

A reaction solution of EXAMPLE 9 was filtered to remove insoluble materials, then the resulting filtrate was passed through a glass column in which 2.72 g of Accurel MP1000 was filled, wherein the Accurel MP1000 had been equilibrated with methanol and distilled water. Ethyl acetate was added to the filtrate passed thus and pH was adjusted to 9, then the layer of ethyl acetate was analyzed by chiral HPLC to obtain the result of (S)-ester : (R)-ester = 95.6 : 1.1 (area % ratio) and ester : acid = 96.7 : 0.87 (area % ratio). In addition, the aqueous layer was acidified to extract with ethyl acetate and the solvent was evaporated, then analysis was performed by chiral HPLC to ascertain that a white (R)-N-(2,6-dimethyl phenyl) alanine was quantitatively obtained at the optical purity of (R)-acid of 97.2% ee and ester : acid = 0.42 : 99.58 (area % ratio).

EXAMPLE 12: Optical resolution of (R), (S)-N-(2,6-dimethyl phenyl) alanine methoxyethyl ester by enzyme and isolation of (R)-N-(2,6-dimethyl phenyl) alanine

24 g of racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine methoxyethyl ester was added to 50 ml of 0.5 M potassium phosphate buffer (pH 7.0) and 1.0 g of Lipase PS was then added thereto, followed by shaking at 200 rpm and 35°C to run a reaction. After the reaction was stopped at 40% conversion, ethyl acetate was added and pH of the aqueous layer was adjusted to 10, then the ethyl acetate layer was partitioned and water was added thereto. The aqueous layer was adjusted to pH 3 – 4, and the ethyl acetate layer was dried over $MgSO_4$ and then filtered. When ethyl acetate was evaporated, 5.3 g of (R)-N-(2,6-dimethyl phenyl) alanine

was obtained at a yield of 57.4%, of which the optical purity was 96% ee when measured by chiral HPLC analysis.

EXAMPLE 13: Esterification of (R)-N-(2,6-dimethyl phenyl) alanine

5.03 g of (R)-N-(2,6-dimethyl phenyl) alanine (96% ee) was dissolved in 15 g of methanol, then 3.25 g (1.05 equivalent) of thionyl chloride was added dropwise thereto at 0°C. After reflux for 2 hrs, when GC analysis was performed, it was ascertained that 2.5% of the (R)-N-(2,6-dimethyl phenyl) alanine remained. Solvent was evaporated and 30 ml of toluene was then added, followed by washing with 50 ml of Na_2CO_3 5 times. The toluene layer was partitioned, followed by drying over $MgSO_4$ to remove the solvent. When (R)-N-(2,6-dimethyl phenyl) alanine methyl ester (4.2 g; isolated yield: 78%) obtained thus was analyzed by chiral HPLC, the optical purity was 97.3% ee. When measured by GC analysis, the chemical purity was 97.2%.

INDUSTRIAL APPLICABILITY

As described above, according to the methods of the present invention, a racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine ester which cannot be enantioselectively resolved by previously known methods because of similar chemical and physical properties can be separated to obtain R-form enantiomer and S-form enantiomer in a simple and low cost process. Accordingly, by the methods of the present invention, (R)- or (S)-N-(2,6-dimethyl phenyl) alanine and its ester compound, being useful as precursors for enantiomerically enriched Metalaxyl, Benalaxyl, Furalaxyl and the like having antifungal activity, can be readily prepared in high optical purity and yield.

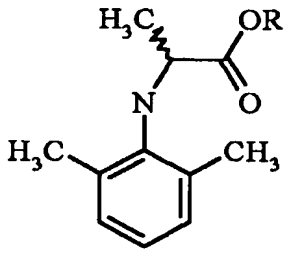
As the present invention may be embodied in several forms without departing from the

spirit or essential characteristics thereof, it should also be understood that the above-described examples are not limited by any of the details of the foregoing description, unless otherwise specified, but rather should be construed broadly within its spirit and scope as defined in the appended claims, and therefore all changes and modifications that fall within the meets and bounds of the claims, or equivalences of such meets and bounds are therefore intended to be embraced by the appended claims.

WHAT IS CLAIMED IS:

1. A method of preparing (R)- or (S)-N-(2,6-dimethyl phenyl) alanine comprising the steps of,

(A) reacting racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine ester as represented in Formula 1 below,



(1)

where

R is selected from the group consisting of unsubstituted or substituted and linear or branched C_1-C_{18} alkyl or alkenyl, unsubstituted or substituted C_3-C_6 cycloalkyl, unsubstituted or substituted aryl alkyl, and unsubstituted or substituted heteroaryl alkyl,

with an effective amount of an enzyme which enantioselectively hydrolyzes one enantiomeric form thereof to produce an (R)-form or (S)-form of N-(2,6-dimethyl phenyl) alanine ("(R)-form alanine" or "(S)-form alanine") and a counter enantiomeric form of N-(2,6-dimethyl phenyl) alanine ester ("counter (S)-form ester" or "counter (R)-form ester"); and

(B) isolating the synthesized (R)-form or (S)-form alanine from a reaction mixture to obtain an optically pure N-(2,6-dimethyl phenyl) alanine.

2. A method of preparing (R)- or (S)-N-(2,6-dimethyl phenyl) alanine ester comprising

the steps of,

(A') reacting racemic (R), (S)-N-(2,6-dimethyl phenyl) alanine ester as represented in Formula 1 above with an effective amount of an enzyme which enantioselectively hydrolyzes one enantiomeric form thereof to produce an (R)-form or (S)-form of N-(2,6-dimethyl phenyl) alanine ("(R)-form alanine" or "(S)-form alanine") and a counter enantiomeric form of N-(2,6-dimethyl phenyl) alanine ester ("counter (S)-form ester" or "counter (R)-form ester"); and

(B') isolating the unhydrolyzed counter (S)-form or (R)-form ester from a reaction mixture to obtain an optically pure N-(2,6-dimethyl phenyl) alanine ester.

3. The method of claim 2, wherein the step (B') is replaced with the following step (B"):

(B") isolating the synthesized (R)-form or (S)-form alanine from a reaction mixture and then esterifying the (R)-form or (S)-form alanine with an alcohol, R-OH, wherein R is the same as in Formula 1, to produce an optically pure N-(2,6-dimethyl phenyl) alanine ester.

4. The method of claim 1, wherein R is selected from the group consisting of allyl, 2-chloroethyl, methoxyethyl and ethoxyethyl.

5. The method of claim 1 or 2, wherein the enzyme is one or more selected from lipases, proteases and esterases derived from microorganisms, animals or plants.

6. The method of claim 5, wherein the enzyme is lipase.

7. The method of claim 5, wherein the enzyme is one or more selected from the group of Lipase AK from *Pseudomonas*, Toyobo Immobilized lipase, Lipoprotein lipase, Lipase PS and AH from *Burkhoderia*, Lipase QLM from *Alcaligenes*, and Lipase OF from *Candida*.

8. The method of claim 1 or 2, wherein a hydrolysis reaction by the enzyme is carried out in an aqueous solution, or a mixed solution containing a small amount of organic solvent.

9. The method of claim 8, wherein the organic solvent is a hydrophilic solvent such as acetone, acetonitrile, alcohol, etc. or a hydrophobic solvent such as isopropyl ether, tert-butyl methyl ether, chloroform, dichloromethane, carbon tetrachloride, hexane, toluene, etc., or mixtures thereof.

10. The method of claim 1 or 2, wherein the hydrolysis reaction is performed at pH 3 – 12 and 0 – 60°C.

11. The method of claim 1 or 2, wherein the isolation is achieved by extracting an unhydrolyzed ester compound ((R)- or (S)-form ester) using organic solvent in the case of the reaction system of only aqueous solution, and partitioning an organic layer containing an unhydrolyzed ester compound to obtain an aqueous layer containing a synthesized enantiomeric alanine in the case of the reaction system of a mixed solution of organic solvent and aqueous solution.

12. The method of claim 1 or 2, wherein the enzyme is an immobilized enzyme on supports or enzyme aggregates crosslinked in any form.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2003/002673

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12P 41/00, C07C 239/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC7 C12P C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean Patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN(CASLINK), eKIPASS, Delphion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nakayama, Hitoshi et al 'A novel chiral microenvironmental probe at the active site of trypsin. Extrinsic Cotton effects of acyl-trypsin possessing an enantiomeric pair of chromophores.' In European Journal of Biochemistry, 112(2), pages 403-409 (1980) see the abstract	1-12
A	Hofstetter, H. et al 'Chiral interaction of a polyclonal anti-dinitrophenyl antibody with dinitrophenyl-amino acids determined by an enantioselective enzyme-linked immunosorbent assay.' In Analytica Chimica Acta, 332(2-3), pages 285-290 (1996) see the abstract	1-12
A	US 4919709 A (Moser, Hans et al) 24 April 1990 see the whole document	1-12

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
27 FEBRUARY 2004 (27.02.2004)	27 FEBRUARY 2004 (27.02.2004)

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR2003/002673

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